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Congenital Non-Spherocytic Hemolytic Anemia

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ABSTRACT

A family with congenital non-spherocytic hemolytic anemia associated with glucose-6-phosphate dehydrogenase (G6PD) deficiency was studied. Two females, heterozygous for the enzyme deficency, had evidence of a hemolytic anemia. The results of chromium-51 erythrocyte life span studies prior to, during, and after periods of primaquine administration suggested that the hemolytic anemia in these women was due to the presence of two populations of red blood cells in their circulation. One population had normal G6PD levels and a normal life span, whereas the other had diminished enzyme activity and a shortened life span.

***In vitro* metabolic studies of the erythrocytes of a heterozygous female and a hemizygous male suggested that, in spite of G6PD deficiency, the synthesis and breakdown of adenosine triphosphate and 2,3-diphosphoglyceric acid was similar to that in normal erythrocytes.**

SOMMAIRE

Les auteurs ont étudié une famille souffrant d'anémie hémolytique congénitale, de nature non sphérocytaire et relevant d'une insuffisance de 6-phosphate glucose déhydrogenase (6PGD). Deux femmes hétérozygotes en ce qui concerne l'insuffisance enzymatique, présentaient des signes d'anémie hémolytique. Les résultats d'une étude sur la longévité des érythrocytes effectuées par le chromium 51, avant, pendant et après administration de primaquine ont permis de croire que l'anémie hémolytique dont souffraient ces femmes était causée par la presence de deux catégories de globules rouges dans leur sang circulant. Une population de globules rouges présentaient une concentration normale de 6PGD et une longévité normale, tandis que l'autre avait une activité enzymatique réduit et une longévité raccourcie.

L'étude *in vitro* du métabolisme des érythrocytes d'une femme hétérozygote et d'un homme hémizygote a permis de supposer que, malgré l'insuffisance de 6PGD, la synthèse et la dégradation du triphosphate d'adénosine et de l'acide 2,3 diphosphoglycérique étaient semblables à celles des érythrocytes normaux.

CONGENITAL non - spherocytic hemolytic anemia is characterized by an intrinsic red cell defect unassociated with any morphological or hemoglobin abnormality. There are numerous reports of one type of this disorder in which the affected males have a moderately severe hemolytic anemia in association with reduced activity of erythrocyte glucose - 6 - phosphate dehydrogenase (G6PD).[2] The disease is transmitted as a sex-linked recessive trait in which the erythrocytes of hemizygotes (affected males) have low enzyme activity and a short life span. The heterozygous females have intermediate enzyme levels and usually do not have clinical disease, although recent reports have suggested that some may have evidence of hemolysis.[2, 12, 21]

In the present study we describe another family with this disorder as well as our investigations of

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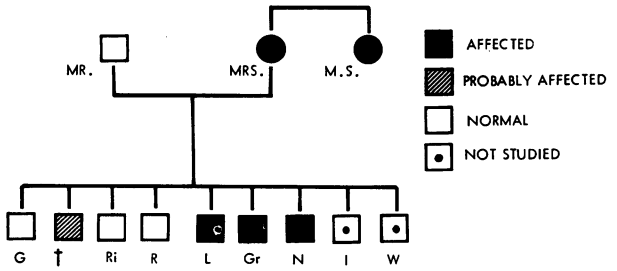

Fig. 1.—The "J" family. "Affected" members have clinical evidence of hemolytic disease. The "probably" affected child was not examined but is reported to have died of "aplastic anemia".

the hemolytic process in both the hemizygous male and the heterozygous female.

CLINICAL MATERIAL

All cases belong to the "J" family (Fig. 1). Three affected and three non-affected boys were studied. The second-born child died at age 4 of "aplastic anemia". A brief history of the affected members follows:

MRS. J. was born in 1930 of Scottish and Cree Indian parents. She recalls no previous severe illness, no jaundice and no anemia. In 1958 she had a cholecystectomy.

L.J. was admitted to the Children's Hospital, Winnipeg, on July 25, 1958, at the age of 4 years because of severe anemia. He was well until four days prior to admission, when he began to vomit and became febrile. Vomiting and fever continued until the day of admission. There was no previous history of jaundice or anemia. On admission the hemoglobin was 3.4 g. %, the reticulocyte count 0.2% and the leukocyte count 22,400/c.mm. The bone marrow was hyperplastic with "maturation arrest" at the early normoblast level. The patient was given 500 ml. of blood on the day of admission and 500 ml. on the second day. His recovery thereafter was uneventful. Subsequent investigation confirmed the diagnosis of congenital nonspherocytic hemolytic anemia. This acute episode would appear to represent an acute aregenerative crisis.

GR.J. was admitted to the Children's Hospital on October 9, 1958, at the age of 27 months. He had been ill for one week with an upper respiratory infection. He was admitted because of severe anemia. Physical examination was negative except for pallor. The hemoglobin on admission was 3.4 g. % and the reticulocyte count 0.3%. The leukocyte count was 15,600/c.mm. with 41% lymphocytes, 47% neutrophils, 9% bands, 2% eosinophils and 1% monocytes. A bone marrow aspiration showed erythroid hyperplasia with an arrest at the late normoblast level. The patient was given 250 ml. of blood on the day of admission and 250 ml. the following day. By October 15 the hemoglobin concentration was 11.6 g. % and the reticulocyte count was 10.9%. Stools were negative for viral pathogens, and no rise in antibody titre to adenovirus type 1 was found (see below).

N.J. was admitted to the Children's Hospital on October 11, 1958 (two days after Gr.J.), because of severe anemia. He too had had an upper respiratory infection and had vomited on the day before admission. The past history was negative; neither jaundice nor anemia had been noted. Physical examination on admission was negative except for pallor. The hemoglobin was 3.0 g. % and the reticulocyte count 0.6%. The leukocyte count on October 12 was 36,000/c.mm. with 30% neutrophils, 1% eosinophils, 54% bands and 15% lymphocytes. He was given 225 ml. of packed red cells and subsequently improved. Adenovirus type 1 was isolated from the stool. Neutralizing antibody titre to adenovirus type 1 was 1/5 on admission; two weeks later it had risen to 1/40.

All members of the family have been well since the hospital admissions described. The additional hematological investigations were performed during 1963.

METHODS AND RESULTS

A. *Hematological Data*

Representative hematological findings in this family are shown in Table I.

TABLE I.—HEMATOLOGIC DATA ON "J" FAMILY

	Mrs. J.	*L.J.*	*Gr.J.*	*N.J.*	*M.S.*	*Mr.J.*	*G.J.*	*Ri.J.*	*R.J.*
Age	32	9	7	5	27	35	15	13	11
Sex	F	M	M	M	F	M	M	M	M
Hb. (g. %)	12.3	11.0	10.4	10.7	11.8	15.2	13.0	12.2	13.5
Reticulocytes (%)	12.2	19.2	16.2	12.2	13.3	1.6	1.5	3.0	1.3
MCV (μ^3)	93	91	116	111					
MCH ($\mu\mu g.$)	35	36	33	30					
MCHC (%)	38	34	29	28					
Bilirubin:									
Indirect (mg. %)	1.6	1.4	1.3	0.3					
Direct	0	0	0.1						

Mrs. J., her sister M.S. and three affected boys have consistently elevated reticulocyte counts and low or low normal hemoglobin concentrations. Their erythrocytes are normal morphologically. The blood of L.J., N.J., G.J. and Mrs. J. was studied for fetal hemoglobin,[7] fetal hemoglobin-containing cells[1] and abnormal hemoglobins by starch gel electrophoresis.[23] No abnormalities were found.

The osmotic fragility of fresh blood and of blood incubated for 24 hours at 37° C. was studied in Mrs. J. and L.J. and was normal. Erythrocyte autohemolysis[8] was normal in Mrs. J. and her three affected sons (Table II).

TABLE II.—PER CENT AUTOHEMOLYSIS* AFTER 48 HOURS' INCUBATION AT 37° C.

	Without glucose	*With glucose*
Control A	2.0	0.29
Control B	0.73	0.63
Mrs. J.	1.6	0.50
L.J.	0.75	0.46
N.J.	0.93	0.38
Gr.J.	0.70	—
Normal range (Dacie[8])	0.4 - 4.5	0.03 - 0.4

*Defibrinated, sterile blood was incubated at 37° C. for 48 hours. The amount of hemolysis was determined as described by Dacie.[8]

B. *Enzyme Studies*

Glucose-6-phosphate dehydrogenase (G6PD) assays were performed on the erythrocytes of the three affected boys and both parents (Table III), using the technique of Zinkham and Lenhard.[25] Enzyme activity could not be detected in the red cells of the three boys. Their mother (Mrs. J.) had a value just below the normal range. Leukocyte G6PD was determined by the technique of Ramot *et al.*[22] The enzyme activity was very low in L.J.'s leukocytes, while that in Mrs. J. was intermediate between the normal and that of her son.

TABLE III.—GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY

	*Erythrocytes**	*Leukocytes***
Normals (12)	140 - 250	77 - 182
Mrs. J.	136	54
L.J.	0	14.5
N.J.	0	
Gr.J.	0	
Mr. J.	146	

* = units/100 ml. RBC.[25]
** = O.D./min./10^9 cells.[22]

C. *Erythrocyte Phosphate Metabolism*

The techniques used for the study of P^{32} incorporation into erythrocyte phosphate compounds within the cell have been described previously.[10]

The red cells of Mrs. J. and L.J. were studied twice. The concentrations of erythrocyte inorganic phosphate (IP) and 2,3-diphosphoglyceric acid 2,3-DPG) were higher than the mean values for five normal adult controls (Table IV). The erythrocytes of these patients were incubated at 37° C. for two minutes in the presence of P^{32}-labelled sodium phosphate. At two minutes the cells were washed and resuspended in a buffered solution containing non-radioactive sodium phosphate. Incubation was continued for an additional 30 minutes. In this way radioactive phosphate entered the cell during the first two minutes and labelled ATP, IP, and 2,3-DPG. The fate of the radioactive "pulse" within the cells could then be followed over the next 30 minutes. In Table V the results of two such studies (a and b) in Mrs. J. and L.J. are recorded along with observations in 10 normal adults. The overall pattern in the patients is similar to that in the normals, except that the initial specific activity of ATP was higher in run "b" in Mrs. J. and L.J. than in the normals. The initial rate of labelling of 2,3-DPG was similar or slightly greater than normal. The further labelling of 2,3-DPG which resulted from the movement of radioactivity from IP and ATP was also similar to the normal.

TABLE IV.—ERYTHROCYTE PHOSPHATE COMPOUNDS (mg. P/100 ml. RBC)

	ATP	*IP*	*2,3-DPG*
Normals: Mean	4.72	2.36	27.2
Range	(2.7 - 6.3)	(.96 - 3.75)	(24.3 - 30.9)
Mrs. J.	6.25	4.64	32.4
L.J.	4.80	4.80	36.4

ATP = adenosine triphosphate; IP = inorganic phosphate: 2,3-DPG = 2,3-diphosphoglyceric acid.

D. *Erythrocyte Cr^{51} Life Span*

Chromium-51 cross-transfusion studies were performed as follows: 20-30 ml. of blood was added to 10 ml. of ACD solution containing 75-125 μc. sodium chromate (Cr^{51}), 120 mg. dextrose, 250 mg. sodium citrate and 80 mg. citric acid. The volume of ACD used was always less than that of blood to avoid the deleterious effect of excess ACD concentration.[19] The first blood sample was drawn 24 hours after infusion and was considered as 100%. The 50% survival (T/2) of Cr^{51}-labelled normal cells as performed in our laboratory is 28-32 days.

(a) Life span studies were performed on L.J. by infusing his Cr^{51}-labelled cells into three hematologically normal recipients. The 50% survival of the infused cells varied from seven to nine days. One of these studies is shown in Fig. 2.

Mrs. J.'s blood was infused into three recipients. The 50% survival in the three studies was 10, 18 and 21 days, respectively. In all three survival curves the pattern of disappearance was that of two populations of cells, one with a T/2 of nine to 18 days and a second with a T/2 which in the three studies varied from 26 to 30 days. One of the three studies is shown in Fig. 3 and demonstrates these two components.

TABLE V.—THE SPECIFIC ACTIVITIES OF ERYTHROCYTE INORGANIC PHOSPHATE (IP), ADENOSINE-TRIPHOSPHATE (ATP), AND 2,3-DIPHOSPHOGLYCERIC ACID (2,3-DPG) FOLLOWING INCUBATION OF WHOLE BLOOD WITH P^{32}—SODIUM PHOSPHATE

Normals	*2 minutes*			*30 minutes*		
	IP	*ATP*	*2,3-DPG*	*IP*	*ATP*	*2,3-DPG*
Mean	100*	92.3	11.95	59.5	69.3	42.2
Range		(72 - 126)	(3.7 - 21.8)	(55.5 - 62.0)	(66.6 - 72)	(40 - 43.5)
Mrs. J.						
a.	100	96.0	15.8	70	67.5	37.3
b.	100	140.0	21.1			
L.J.						
a.	100	124.0	21.8	74	57	40.5
b.	100	156.0	32.4			

*All results are expressed as a percentage of the specific activity of intracellular inorganic phosphate at two minutes, which is arbitrarily taken as 100%. Two separate studies, (a) and (b), are shown for each of the patients.

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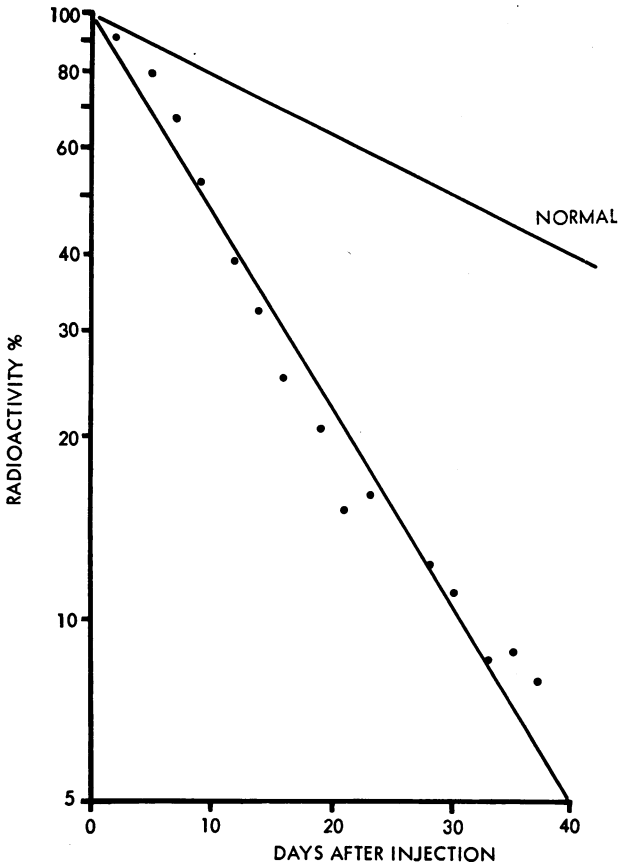

Fig. 2.—Chromium-51 red cell life span of L.J.'s cells in a normal recipient. A normal survival curve with a T/2 of 30 days is included for comparison.

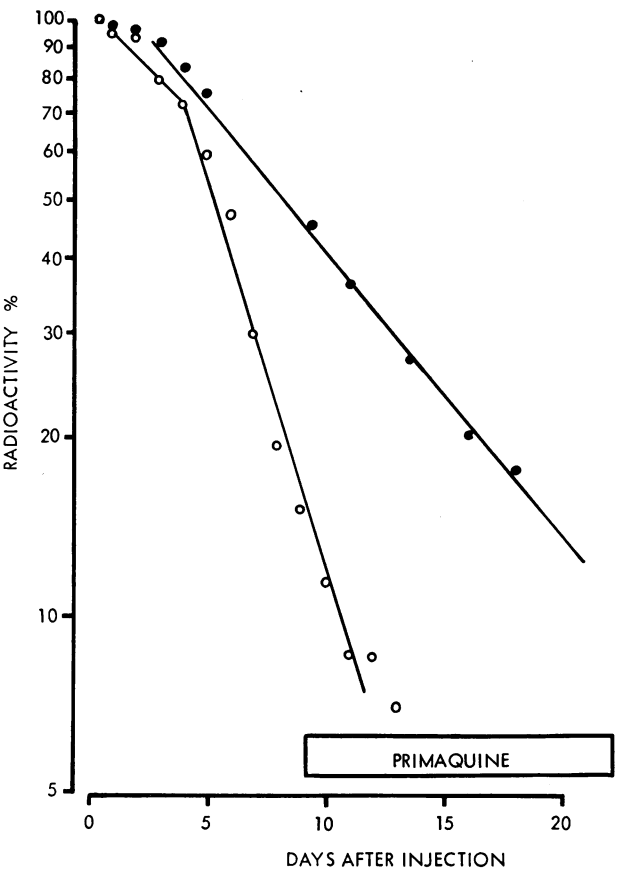

Fig. 4.—Chromium-51 red cell survival of L.J.'s cells in two normal adult recipients. One recipient received no medication (closed circles) whereas the other (open circles) received primaquine 30 mg./day orally beginning on day 4.

(b) The possibility raised by the above studies was that the blood of Mrs. J. contained two populations of erythrocytes, one with little or no glucose-6-phosphate dehydrogenase activity and a short life span and one with normal levels and a normal life span.

To test this possibility a second series of cross-transfusion studies was performed. Initially the blood of L.J. was infused into two recipients. One served as a control and again the T/2 was eight to nine days. Four days after infusion the other recipient was given primaquine, 30 mg. per day (Fig. 4). Coincident with the administration of primaquine, there was a rapid fall in circulating radioactivity. The blood of Mrs. J. was then infused into two recipients. One recipient was started on primaquine 19 days after infusion, and it is evident that the life span of the cells was not shortened by the drug (Fig. 5). The second was

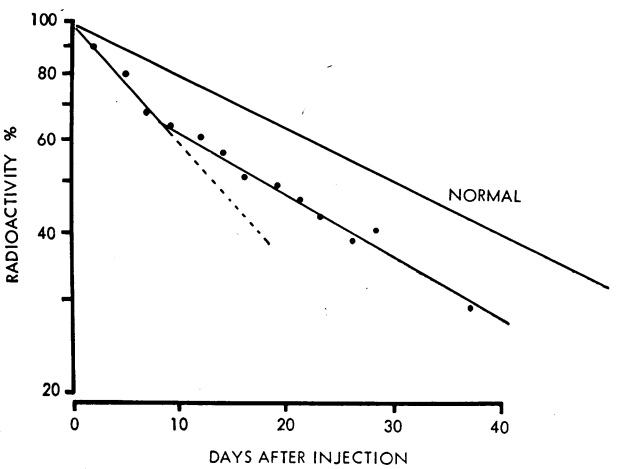

Fig. 3.—Chromium-51 red cell life span of Mrs. J.'s cells in a normal recipient. A normal survival curve with a T/2 of 30 days is included for comparison.

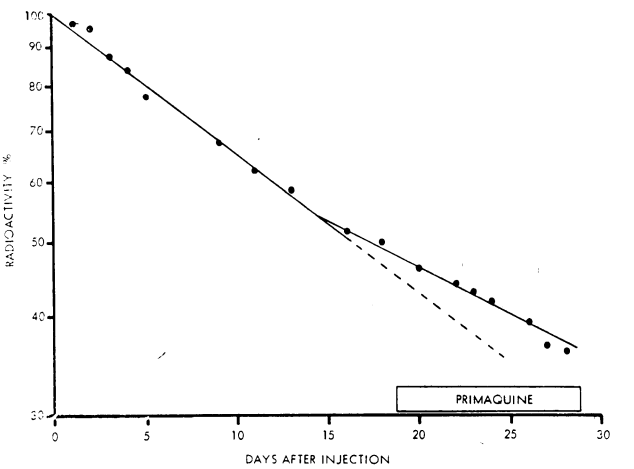

Fig. 5.—Chromium-51 red cell survival of Mrs. J.'s cells in a normal adult recipient. Primaquine (30 mg./day) was given orally from day 19.

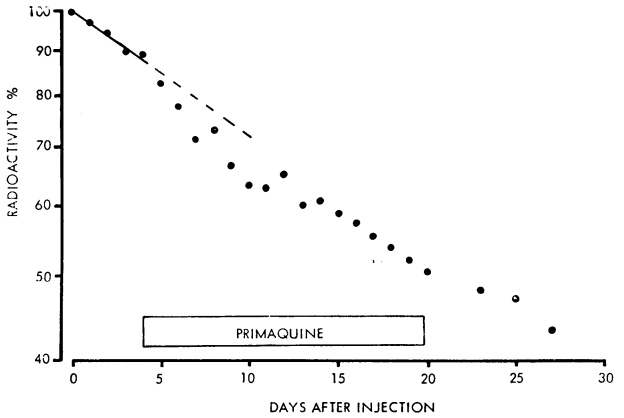

Fig. 6.—Chromium-51 red cell survival of Mrs. J.'s cells in one normal recipient who received primaquine (30 mg./day) orally from day 4 to day 20.

treated with primaquine from day 4. It appears that in this recipient the rate of red cell destruction may have been accelerated for a few days but thereafter primaquine was without effect on the red cell life span (Fig. 6).

Discussion

The affected members of the "J" family suffer from a chronic hemolytic state due to an inherited intrinsic defect of their red blood cells. There is no other associated disease, no abnormality in the shape of the cells and none in their hemoglobin. Thus their disorder can be classified as congenital non-spherocytic hemolytic disease. The autohemolysis data (Table II) together with the relatively mild clinical course are similar to those in cases referred to by Dacie[8] as type I. All three affected males have had severe aregenerative crises, and it is likely that the child who died also suffered from the same disease. There is no history of crisis in the mother or the aunt.

The affected males in the family have a deficiency of erythrocyte and leukocyte glucose-6-phosphate dehydrogenase (G6PD). In these families the enzyme defect is thought to be responsible for the shortened red cell life span, and recent studies in several families have indicated that the enzyme present in these cells is abnormal in structure.[11, 13]

The red cell content of IP and 2,3-DPG in Mrs. J. and L.J. was slightly higher than the normal. This may be related to the high percentage of young red cells and reticulocytes, since such cells have higher concentrations of these compounds.[3, 14] Our findings are similar to those of others who were unable to demonstrate low levels of ATP in the cells of patients with this disorder.[15, 20]

Although it is agreed that resting ATP levels in the erythrocytes in this condition are normal, these cells, upon exposure to oxidants, show a greater fall in ATP than do normal cells.[15, 21] It has been suggested that this is due to a decreased rate of ATP synthesis under these conditions. Our studies indicate that in the fresh, non-incubated cell the pattern of ATP synthesis (as evidenced by P^{32} incorporation) is similar to, if not more rapid than, normal. It seems likely, therefore, that the rapid decline of red cell ATP during incubation with oxidants observed by others is not related to a primary abnormality in ATP synthesis or breakdown within these cells.

In those families completely studied it is evident that inheritance of G6PD is a sex-linked recessive trait. It would be expected, therefore, that the enzyme levels in the cells of Mrs. J. (a heterozygote) should be intermediate between the normal and the very low levels found in her sons. This is the case in the leukocytes of these subjects. However, the erythrocytes of Mrs. J. are near normal in activity, although the erythrocytes of her three affected sons had no G6PD activity as determined by the techniques employed. The relatively high value (near normal) in her erythrocytes presumably is related to the young population of erythrocytes in her peripheral blood, since G6PD levels are higher in young erythrocytes.[17] It is surprising therefore that Mrs. J. and her sister have evidence of chronic hemolysis. Negro or Caucasian males with glucose-6-phosphate dehydrogenase deficiency (primaquine sensitivity and favism,[18] respectively) have much lower concentrations of enzyme activity, yet have normal or near-normal red cell life spans.[6]

One possible explanation for the short erythrocyte life span in Mrs. J. is suggested by the studies of Lyon,[16] who has proposed that every cell of a female has within it only one active X chromosome. The second X of each cell is inactive and forms the hyperpyknotic sex body. She further states that it is chance which determines which of the two X's will be active in a particular cell. This hypothesis has also been advanced by Beutler, Yeh and Fairbanks[4] and has received support from several recent studies.[5, 9, 24] This theory would predict that in Mrs. J. there are two populations of erythrocytes, one with normal glucose-6-phosphate dehydrogenase activity and the other with activity similar to that of her sons.

The life span data (Fig. 3) are consistent with the hypothesis that she has two cell populations. Additional support for this is found in the primaquine studies (Fig. 5) in which it was shown that Mrs. J.'s long-lived cells do not have an increased susceptibility to primaquine and therefore despite their age are not grossly deficient in G6PD activity. When it was started four days after the cells had been transfused (Fig. 6), primaquine may have accelerated hemolysis of the short-lived cells for a few days but was without effect on the remaining cells.

It should be noted that the appearance of a hemolytic anemia in the heterozygous female carrier of type I non-spherocytic hemolytic anemia is not unique to this family. This has been reported previously,[2, 12, 21] and it has been suggested that

this situation could be explained by the Lyon hypothesis. It should be pointed out, however, that there are many families in which the female heterozygotes have no evidence of a hemolytic anemia. The variable manifestations may be due to the percentage of cells with the mutant gene on the functional X chromosome. If this is high, the patient will suffer from a hemolytic anemia; if low, there may be no detectable shortening of erythrocyte life span.

SUMMARY

A family with congenital non-spherocytic hemolytic anemia associated with deficient erythrocyte glucose-6-phosphate dehydrogenase activity is reported. The synthesis and turnover of adenosine triphosphate and 2,3-diphosphoglyceric acid were similar in glucose-6-phosphate dehydrogenase-deficient and in normal erythrocytes. Evidence is presented which suggests that the blood of the heterozygous mother contains two populations of erythrocytes: one with normal activity of glucose-6-phosphate dehydrogenase and a normal life span and the other with deficient enzyme activity and a shortened life span.

We wish to thank Miss E. J. Brown for the determinations of leukocyte enzyme activity.

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